Kidney International, Vol. 59 (2001), pp. 1342–1353

Selenium-deficient diet induces renal oxidative stress and injury via TGF-β1 in normal and diabetic rats

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Background. Oxidative stress has been implicated in the pathogenesis of diabetic nephropathy. Although glucose itself can initiate oxidative stress, deficiency of essential trace elements such as selenium (Se) may exacerbate this oxidative stress in diabetic rats. The mechanism by which Se deficiency causes oxidative stress and renal injury is not completely understood. This study tested the hypothesis that Se deficiency induces renal oxidative stress and renal injury via transforming growth factor-β1 (TGF-β1).

Methods. Fifty-four male Wistar rats were used. Diabetes was induced in 27 rats by streptozotocin, and the other 27 rats received buffer only. Ten weeks after induction of diabetes, both normal and diabetic rats were killed, their kidneys removed, and glomeruli were isolated. Glomeruli from normal and diabetic rats were incubated in the presence of TGF-β1 alone or its neutralizing antibody. Antioxidant enzyme (Cu-Zn) superoxide dismutase (Cu-Zn SOD), catalase, and glutathione peroxidase (GSH-Px) activities; total glutathione; and lipid peroxidation were determined. For Se studies, 15 normal and 15 diabetic rats were divided into groups of five each and fed either a regular, Se-deficient, or Se-supplemented diet one week after induction of diabetes. Ten weeks after feeding these diets, rats were killed and glomeruli were isolated. Oxidative stress was examined by determining the mRNA expressions for antioxidant enzymes and also for TGF-β1. Plasma glucose and albuminuria were determined. Histology of the kidney and interlobular artery was evaluated by light microscopy.

Results. In vitro studies showed that TGF-β1 significantly reduced glomerular catalase and GSH-Px activities as well as total glutathione levels with an increase in lipid peroxidation in both normal and diabetic rats. Antibody to TGF-β abrogated these changes. There was no effect of TGF-β1 on Cu-Zn SOD. Like TGF-β1, a Se-deficient diet caused a significant decrease in glomerular mRNA expression for Cu-Zn SOD, catalase, and GSH-Px, but a significant increase in TGF-β1 mRNA expression. Also, a Se-deficient diet caused an increase in albuminuria, glomerular sclerosis, and plasma glucose levels in both normal and diabetic rats. The deficient diet caused a decrease in the lumen size of the interlobular artery. Se supplementation to diabetic rats up-regulated mRNA expression for antioxidant enzymes, and significantly reduced but did not normalize that of TGF-β1. Glomerular sclerosis was normalized and the interlobular artery lumen size was greatly enlarged in diabetic rats by Se supplementation. Also, the tubulointerstitium was preserved by Se supplementation in diabetic rats.

Conclusions. The data show that TGF-β1 is a pro-oxidant and Se deficiency increases oxidative stress via this growth factor. In addition, Se deficiency may simulate hyperglycemic conditions. Se supplementation to diabetic rats prevents not only oxidative stress but renal structural injury, as well.

The pathology of the kidney in diabetes mellitus is characterized by thickening of the glomerular and tubular basement membranes and accumulation of basement membrane-like material in the mesangium [1]. The mesangium expands gradually with duration of diabetes and becomes the dominant pathologic process causing occlusion of the glomerular capillary lumen. Patients with marked mesangial expansion demonstrate albuminuria and glomerular hypertension. Studies have shown that the albumin excretion rate of between 20 mg and 300 mg/day, called microalbuminuria, is a predictor of glomerular disease in diabetic patients, and its prevention prolongs the onset of renal failure [2, 3]. Several studies have implicated reactive oxygen species (ROS) or oxidative stress as one of the important causes of proteinuria [4–7]. Hyperglycemia is probably the single most important cause of increased oxidative stress in diabetic animals and humans, because glucose itself can initiate ROS production [8, 9]. In its enediol form, glucose is prone to transition metal-catalyzed autoxidation, yielding hydrogen peroxide, hydroxyl radical, and ketoaldehyde. Oxidative stress implies an overloading of "oxidants" or ROS that damage a cell. In diabetes, oxidative stress seems mainly due to both an increased production of plasma or tissue ROS concentrations [10–16]. Three primary antioxidant enzymes have been demonstrated in mammalian systems [4, 14]: superoxide dismutase (SOD), catalase, and gluta-

Key words: kidney injury, diabetic nephropathy, trace element, albuminuria, glomerulosclerosis, antioxidant, hyperglycemia.

Received for publication January 26, 2000
and in revised form October 18, 2000
Accepted for publication October 23, 2000

thione peroxidase (GSH-Px). SOD exists as Cu-Zn SOD and Mn SOD. Cu-Zn SOD, which occurs predominantly in the cytosol and nucleus, converts superoxide radical into hydrogen peroxide, which is then converted to water by both catalase and GSH-Px. Thus, the three enzymes prevent tissue damage by detoxifying ROS. Besides these three scavenger enzymes, several endogenous and exogenous antioxidants, such as glutathione, vitamins E, and C, reduce oxidative burden and maintain normal equilibrium. The available evidence indicates a decrease in tissue and circulating levels of these antioxidant enzymes and glutathione in diabetic animals and human subjects [10, 14, 15], and thus increased oxidative stress in diabetes.

Selenium (Se) is an essential trace element, and its clinical importance in various pathophysiologic conditions has been increasingly recognized [17–20]. Se is an integral component at the catalytic sites of the enzyme GSH-Px. Deficiency of Se causes a profound reduction in the activity of GSH-Px in several tissues, particularly in the liver [21–23], resulting in increased oxidative stress. Se has insulin-like effects because Se supplementation has been shown to reduce hyperglycemia and improve glucose tolerance in diabetic rats without any effect on endogenous insulin levels [24–28]. Also, the addition of sodium selenate to rat adipocytes stimulated glucose transport [29]. These in vitro insulin-like effects are in agreement with the observation that insulin-stimulated glucose oxidation was decreased in adipocytes from Se-deficient rats [30]. Taken together, all of these studies suggest that Se improves glucose metabolism, and Se deficiency causes a physiologic condition similar to hyperglycemia.

Selenium deficiency may simulate hyperglycemic conditions in other aspects as well. For example, Se deficiency has been shown to cause proteinuria in normal rats [31–33]. This was substantiated by another study in which proteinuria induced in rats by aminonucleoside of puromycin was prevented by Se and vitamin E supplementation [34]. Rats fed a diet deficient in both Se and vitamin E for 16 weeks demonstrated severe interstitial disease with an increase in collagen deposition [31]. In diabetic rats, Se supplementation reduced not only proteinuria [27, 35] but glomerular sclerosis as well [35]. This is consistent with a human study in which Se supplementation reduced albuminuria in patients with diabetic microvascular complications [36]. Another similarity between Se deficiency and hyperglycemia is that both conditions are associated with up-regulation of renal transforming growth factor-β1 (TGF-β1) mRNA expression [37, 38]. Lipid peroxidation, which is another mechanism for oxidative cell damage, is also increased in both conditions [38–45]. Thus, there is compelling evidence to implicate Se deficiency or its supplementation in the pathophysiology of renal complications in both diabetic and nondiabetic conditions.

Transforming growth factor-β1 is the prototype of a polypeptide regulatory factor [46–53]. TGF-β1 is multifunctional, and depending on the metabolic status of the cell, it can either stimulate or inhibit the same process. One of the important functions of TGF-β1 is its effect on the extracellular matrix proteins such as collagen, proteoglycans, fibronectin, and laminin [46–53]. TGF-β1 has the following effects on the extracellular matrix proteins. (*1*) It stimulates the synthesis more than the degradation of extracellular matrix proteins, particularly collagen, by mesangial cells [54]. (*2*) It regulates enzymes that degrade extracellular matrix proteins [55, 56]. (*3*) It increases the expression of cell adhesion molecules (integrins) of matrix proteins [57]. Collectively, these processes result in enhanced accumulation of extracellular matrix components, which is beneficial for wound healing but deleterious to the glomerulus. The latter becomes sclerosed as in diabetes or other glomerular diseases in response to TGF-β1. Because of this function, TGF-β1 is considered a profibrotic cytokine.

How Se deficiency causes proteinuria and glomerular sclerosis is not completely understood. In this study, we examined the possibility that Se deficiency causes increased oxidative stress via TGF-β1 in normal and streptozotocin (STZ) diabetic rats. Although Se deficiency causes tubulointerstitial disease and glomerular sclerosis, it is not known whether this dietary deficiency also induces changes in the structure of the renal arteries. The objectives of this study were therefore several-fold: (*1*) to examine whether TGF-β1 induces oxidative stress in normal and diabetic glomeruli; (*2*) to investigate whether Se deficiency increases glomerular oxidative stress and TGF-β1 mRNA expression as well as proteinuria in normal and diabetic rats; (*3*) to determine whether Se deficiency causes any structural changes in the tubulointerstitium and renal arteries; and (*4*) to test whether Se supplementation improves oxidative stress and renal injury in diabetic rats. To our knowledge, studies involving tubulointerstitium and renal arteries in rats fed either a Se-deficient or supplemented diet in diabetic rats have not been reported.

METHODS

Reagents

All reagents were purchased either from Sigma Chemical Co. (St. Louis, MO, USA), Ambion, Inc. (Austin, TX, USA), or R&D Systems (Minneapolis, MN, USA).

Animals

A total of 54 male Wistar rats weighing 80 to 100 g was used. Diabetes was induced in 27 animals by a single intraperitoneal injection of STZ (65 mg/kg) in 0.1 mol/L citrate buffer, pH 4.5. An equal number of control rats received injections of buffer only. Diabetes was con-

Table 1. Composition of selenium-deficient diet

Ingredients	g/kg	%
Torula yeast	300	30
Sucrose	590	59
Corn oil	50	5
DL-methionine	3	0.3
Calcium carbonate	12	1.2
Vitamin mixture (Teklad 40060)	10	1.0
Mineral mixture (Teklad 80313 with Se omitted)	35	3.5

firmed (blood glucose >200 mg/dL) in whole blood obtained from the tail vein, using the glucose reagent strips supplied by Sigma Chemical Co. To accomplish the first objective, 12 normal and 12 diabetic rats were used. Ten weeks after induction of diabetes, both normal and diabetic rats were sacrificed under pentobarbital anesthesia (5 mg/100 g). Kidneys were excised and glomeruli were isolated, as described by a previous method [58].

To accomplish the other objectives, 15 normal and 15 diabetic rats were divided into groups of 5 rats each and fed a basal (regular) diet containing 0.27 mg/kg Se, Se-deficient diet (<0.025 mg/kg), or Se-supplemented diet (0.78 mg/kg) for 10 weeks. These diets were started one week after establishment of diabetes. Both Se-deficient and regular diets were supplied by Bio-Serv (Frenchtown, NJ, USA). The composition of the Se-deficient diet is shown in Table 1. Se is added as sodium selenite. This synthetic diet provided the following percentages: 15.05% protein, 6.45% fat, 2.89% fiber, 1.03% moisture, and 69.20% carbohydrate. All diets were isocaloric and provided 3.958 kcal/g. All rats were allowed to eat and drink tap water ad libitum. At the end of the study, each rat was placed in a metabolic cage, and a 24-hour consumption of food and water as well as urine volume was collected.

Effect of TGF-β1 on glomerular antioxidant enzymes, glutathione, and lipid peroxidation

Glomeruli from two to three rats were pooled and incubated for six hours in the presence of TGF-β1 (5 and 10 ng/mL) or TGF-β1 plus antibody to TGF-β (5 and 10 ng/mL) or antibody alone (5 ng/mL) for determinations of antioxidant enzymes, glutathione, and lipid peroxidation. The neutralizing antibody was a polyclonal antibody, which is a cocktail of recombinant TGF-β1, porcine TGF-β1–2, porcine TGF-β2, and rat TGF-β5. The viability of glomeruli for six hours in the incubation medium was documented by linear increase in one of the antioxidant enzyme (catalase) activities from 2 to 12 hours. Enzyme activity (U/mg protein) at 2, 4, 6 and 12 hours was 7.80 ± 0.2, 16.92 ± 1.14, 44.17 ± 2.05, and 74.00 ± 2.60 (mean ± SEM), respectively.

Preparation of tissue extracts for enzyme assays

Glomeruli were homogenized in 10 volumes of cold Triton × 100 (0.2%, vol/vol) for SOD and GSH-Px activities, and a 20% glomerular homogenate was prepared in 25 mmol/L KH_2PO_4-NaOH buffer, pH 7.0, for catalase activity. The homogenates were centrifuged at 12,000 × *g* for 10 minutes, and the supernatants were used for enzyme assays, as described previously [59]. Protein concentration was determined in these supernatants by the method of Lowry et al [60].

Enzyme assays

Superoxide dismutase activity was measured by the xanthine-xanthine oxidase cytochrome C method, as described previously [59]. The standard assay mixture (250 μL) contained 20 mmol/L potassium phosphate buffer, pH 7.8, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2 mmol/L ferricytochrome C, 50 mmol/L xanthine, and 10 mmol/L fresh potassium cyanide. The reaction was initiated with sufficient xanthine oxidase to cause an increase in absorbance of 0.025 per minute at 550 nm. Total SOD was determined in 0.2% Triton × 100 glomerular supernatant (20 μL) and Cu-Zn SOD in $CHCl_3$/methanol extract. CU-Zn SOD was calculated as the difference between total SOD and Mn-SOD. One unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of the reaction under standard conditions. The enzyme activity was expressed as units per milligram of protein.

Catalase activity was assayed by the method of Johansson and Håkan Borg [61]. The method is based on the reaction of catalase from tissue sample with methanol in the presence of hydrogen peroxide. The formaldehyde produced was measured with Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chromogen. The reaction mixture contained in a total volume of 210 μL: 50 μL 250 mmol/L buffer, 50 μL 100% methanol (wt/vol), 10 μL 27% hydrogen peroxide (wt/vol), and 100 μL glomerular supernatant and incubated with continuous shaking for 20 minutes at room temperature. The reaction was terminated by addition of 50 μL 7.8 mol/L KOH followed by addition of 100 μL 34.2 mmol/L purpald and continuous shaking for 10 minutes. To obtain a colored compound, 50 μL 65.2 mmol/L potassium periodate were added, and the sample centrifuged to precipitate any particulate material. The absorbance of the clear supernate was measured at 550 nm in a spectrophotometer. Catalase activity was expressed as units/mg protein.

Glutathione peroxidase activity was measured by the procedure of Paglia and Valentine [62] as modified by L'Abbe and Trick [63], in which the oxidation of GSH and NADPH are coupled in the presence of glutathione reductase. The reaction mixture contained in a total volume of 250 μL: 150 nmol/L potassium phosphate buffer,

pH 7.0, 5 mmol/L EDTA, 0.5 mmol/L sodium azide, 2 mmol/L GSH, 0.26 mmol/L NADPH, and 1 U/mL glutathione reductase and glomerular supernatant (1.25 μL). The reaction was initiated by adding 1.2 mmol/L t-butyl hydroperoxide as substrate. One unit of enzyme activity was defined as the oxidation of 1.0 μmol of NADPH/min in the system described and expressed as mU/mg protein.

Total glutathione and lipid peroxidation

Immediately after isolation of glomeruli, a 5% homogenate was made with 5% sulfosalicylic acid and centrifuged. Total tissue glutathione was determined by the DTNB [5-5′-dithiobis (2-nitrobenzoic acid)]-glutathione reductase recycling assay as described by Anderson [64]. Total glutathione content was expressed as nmol/mg protein. Lipid peroxidation was assessed by the production of malonylaldehyde, using thiobarbituric acid [40], and expressed as nmol of thiobarbituric acid reactive substances/mg protein.

Quantitation of mRNAs for antioxidant enzymes and TGF-β1

Glomeruli were lyzed and denatured by 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% sarcosylm and 0.1 mol/L 2-mercaptoethanol. Total RNA was extracted by the method of Chomczynski and Sacchi [65]. Twenty micrograms of total RNA were used for each assay. Rat Cu-Zn SOD (nucleotides 50 to 429), catalase (nucleotides 588 to 1011), and GSH-Px (nucleotides 29 to 345) cDNAs were kindly provided by Dr. Jonathan L. Tilly (Massachusetts General Hospital, Boston, MA, USA) and rat TGF-β1 (nucleotides 601 to 1586) by Dr. Michael B. Sporn (National Institutes of Health, Bethesda, MD, USA). The plasmid DNAs cloned in pGEM 4Z vector were linearized with BamHI enzyme, and antisense RNA probes were generated by T7 RNA polymerase using α-^{32}P-UTP (800 Ci/mmol). Full-length transcripts were recovered by running a 5% urea acrylamide gel. Electrophoretically eluted transcripts were ethanol precipitated and used for hybridization. Each labeled riboprobe (2×10^{-4} to 8×10^{-4}; Promega, Madison, WI, USA) was hybridized with total RNA overnight at 37°C and protein was removed by using sarcosyl and proteinase K digestion (2 g). The hybrid RNA was extracted in TE saturated phenol:chloroform and precipitated with absolute alcohol. The precipitate was diluted in 10 μL of loading buffer and preheated for three to five minutes at 95°C before loading on to a 5% urea acrylamide gel. The gel was run at 250 V for one hour and exposed to a Fuji film overnight, and autoradiograms were developed. β-Actin mRNA was used as an internal control. p-TRI-β-actin plasmid DNA containing 126 bp cDNA fragment of rat β-actin gene (nucleotides 2682 to 2779) was purchased from Ambion, Inc. The plasmid DNA was linearized with Xbal enzyme, and suitable RNA polymerase was used to synthesize antisense β-actin RNA probe.

Urinary albumin and plasma glucose

Urinary albumin concentration was determined by the radioimmunoassay method of Brodows et al [66] and plasma glucose by the glucose oxidase method, using the reagents supplied by Sigma Chemical Co.

Histology of the kidney

Coronal sections of the kidney (2 μm thick) were stained with periodic acid-Schiff and examined by light microscopy in a blinded fashion to the animal group. The following measurements were made.

Glomerular morphometry

In each animal, 20 glomeruli were examined for glomerular volume and fractional mesangial area by digital image analysis using the software Image-proPlus (version 3.0) system developed by Media Cybernetics (Media Cybernetics, Silver Spring, MD, USA). Instrumentation consisted of a microscope, a microscope-mounted CCD camera (768 × 493 resolution), and an IBM computer with a color video screen for projecting and manipulating the images. Images of glomeruli at ×400 magnification were digitized, presented in pseudo-color, and saved. The digitized images were then projected on the computer screen and analyzed at a resolution of 768 × 493 pixels. The glomerular area or volume, defined as the cross-sectional area (CSA) of the renal corpuscle bounded by the Bowman's capsule, was determined by manually outlining the Bowman's capsule on the image screen using the cursor, and the area was automatically calculated by the computer. The area of the mesangial matrix was measured by pseudo-color image to aid visualization of the mesangium. The fractional mesangial area was expressed as a percentage of the glomerular area.

Morphometry of interlobular artery

Sections stained with periodic acid-Schiff were examined for various characteristics of the artery [67]. Measurements of morphometric parameters were performed by digital image analysis, as described previously in this article. The interlobular artery was identified as a single muscular artery within the inner cortex and at times lying close to the glomerulus. Arteries that were not sectioned transversely (that is, wall thickness was asymmetrical) were excluded from the study. Therefore, two to five arteries from each animal were evaluated. Images of the arteries at ×400 magnification were digitized and saved. The digitized images were then projected on the computer screen and analyzed at a resolution of 768 × 493 pixels. The total CSA (CSA_{tot}) of the artery, defined as the CSA of the lumen plus the vessel wall, was deter-

Table 2. Effect of transforming growth factor-β1 (TGF-β1) on antioxidant enzymes, total glutathione and lipid peroxidation (TBARS) in glomeruli from normal (N) and diabetic (D) rats

Parameter	Rats	Control	TGF-β_1		TGF-β_1 + TGF-β antibody		TGF-β antibody without TGF-β_1
			5 ng/mL	*10 ng/mL*	*5 ng/mL*	*10 ng/mL*	*5 ng/mL*
Cu-Zn SOD	N	26.37 ± 3.10 (6)[a]	25.32 ± 2.06 (6)	27.66 ± 2.41 (6)	27.39 ± 2.48 (6)	24.70 ± 2.95 (6)	21.16 ± 2.16 (4)
U/mg protein	D	17.42 ± 2.01 (6)	17.98 ± 1.55 (6)	17.99 ± 1.38 (6)	16.12 ± 1.70 (6)	17.73 ± 0.80 (6)	16.65 ± 1.02 (4)
Catalase	N	57.95 ± 1.88 (6)[a]	37.71 ± 1.87 (6)[b]	41.21 ± 1.39 (6)[b]	53.42 ± 1.06 (5)	56.07 ± 1.57 (5)	59.80 ± 1.62 (4)
U/mg protein	D	35.33 ± 1.14 (6)	24.26 ± 1.32 (5)[d]	25.63 ± 0.83 (5)[d]	33.38 ± 0.73 (5)	36.28 ± 1.29 (5)	34.48 ± 0.57 (5)
Glutathione Px	N	231.21 ± 11.91 (4)[a]	171.83 ± 7.91 (6)[b]	187.30 ± 6.99 (5)[c]	207.35 ± 15.12 (4)	213.82 ± 6.00 (4)	230.56 ± 11.30 (4)
mU/mg protein	D	128.17 ± 4.57 (5)	119.70 ± 8.48 (6)	100.93 ± 2.11 (4)[d]	134.46 ± 3.60 (4)	125.29 ± 10.80 (5)	129.40 ± 4.92 (4)
Glutathione	N	31.67 ± 1.36 (5)[a]	30.66 ± 1.63 (6)	27.24 ± 1.04 (4)	29.40 ± 1.10 (6)	25.93 ± 0.41 (6)	29.00 ± 1.09 (4)
nmol/mg protein	D	22.60 ± 1.16 (6)	22.00 ± 1.31 (6)	16.43 ± 1.04 (4)[e]	20.48 ± 1.50 (5)	21.53 ± 0.79 (5)	21.00 ± 0.83 (4)
TBARS	N	1.94 ± 0.08 (6)[a]	1.89 ± 0.06 (4)	2.49 ± 0.12 (6)[c]	2.17 ± 0.09 (4)	2.07 ± 0.15 (6)	2.09 ± 0.08 (4)
nmol/mg protein	D	2.32 ± 0.05 (6)	2.30 ± 0.09 (6)	2.82 ± 0.18 (5)[e]	2.38 ± 0.10 (6)	2.49 ± 0.10 (6)	2.47 ± 0.14 (4)

Each value represents the mean ± SEM. Numbers in parentheses represent number of determinations.
Abbreviations are: TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; Px, peroxidase.
[a] Normal vs. diabetic, $P < 0.05$–0.005 (comparison includes controls as well as TGF-β_1 and its antibody)
[b] Control vs. TGF-β_1 (5 ng/mL), $P < 0.001$
[c] Control vs. TGF-β_1 (10 ng/mL), $P <$ 0.0.025-0.001
[d] Control vs. TGF-β_1 (5 or 10 ng/mL), $P < 0.001$
[e] Control vs. TGF-β_1, $P < 0.01$ (glutathione) or $P < 0.02$ (TBARS)

mined by manually outlining the external circumference of the vessel on the image screen using the cursor, and the area was automatically calculated by the computer. Then, the luminal CSA (CSA_{lum}) was determined by giving pseudo-color to the lumen image, and the area was calculated by the computer. The short and long internal diameters of the vessels, defined as the shortest and longest distances between the two perpendicular lines across the vessel from one adluminal side of the internal elastic lamina to the other, were measured.

The media CSA of the vessel was calculated as:

$$CSA = (CSA_{tot} - CSA_{lum}) \times (ID_{short}/ID_{long})$$

where CSA_{tot} and CSA_{lum} are the total (lumen plus vessel wall) and luminal CSAs, respectively, and ID_{short} and ID_{long} are the short and long internal diameters, respectively. The external diameter (ED) was determined from:

$$ED = 2 \times (CSA_{tot} \times ID_{short}/ID_{long}/\pi)^{1/2}$$

Media thickness was determined from $(ED - ID_{short})/2$, and the media-lumen ratio from $(ED - ID_{short})/2/ID_{short}$.

Tubulointerstitium

The pathology of the tubulointerstitium was evaluated semiquantitatively for the presence of (*1*) tubular dilation, (*2*) tubular atrophy and disrupted tubular basement membrane, (*3*) interstitial fibrosis, and (*4*) interstitial inflammatory cell infiltrates [68].

Statistical analysis

Multiple-group comparisons were analyzed by one-way analysis of variance. When significant *F* values were noted among groups, post hoc analyses were performed using Tukey's test. Student *t* test was used to calculate the significance between normal and diabetic rats. Results are expressed as mean ± SEM; a *P* value < 0.05 was considered significant.

RESULTS

The effects of TGF-β1 and neutralizing antibody to TGF-β on antioxidant enzymes, total glutathione, and thiobarbutiric acid reactive substances (TBARS) in normal and diabetic glomeruli are shown in Table 2. As evident, the enzyme activities of Cu-Zn SOD, catalase, and GSH-Px were significantly lower in diabetic than in normal rats. Similarly, the glomerular glutathione concentration was significantly lower in diabetic rats. In contrast, the lipid peroxidation, as measured by TBARS, was significantly higher in diabetic than in normal rats. TGF-β1 has no effect on Cu-Zn SOD either in normal or diabetic glomeruli. TGF-β1 either at 5 or 10 ng/mL significantly lowered catalase and GSH-Px activities as well as glutathione levels and increased lipid peroxidation in both normal and diabetic glomeruli. These changes induced by TGF-β1 were abrogated by its neutralizing antibody in either the presence or absence of TGF-β1.

Table 3 shows general information on normal or diabetic rats fed either a regular, Se-deficient, or Se-supplemented diets for 10 weeks. As evident, the body weight was significantly lower ($P < 0.001$) in diabetic compared with normal rats. In general, the kidney weight, 24-hour food intake, water intake, urine volume, and albuminuria were significantly higher in diabetic than in normal rats. Plasma glucose levels were also higher in diabetic rats. No differences were observed in food intake, water intake, and urine volume among normal or diabetic groups. However, Se deficiency caused a significant increase in

Table 3. General information on rats fed either a regular, selenium-deficient (Se−) or selenium-supplemented (Se+) diet at the time of killing

	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Parameter	Regular		Se−		Se+	
Body weight *g*	537 ± 22[a]	364 ± 15	510 ± 32	397 ± 20	496 ± 27	387 ± 21
Kidney weight *g*	3.71 ± 0.4[a]	6.18 ± 0.37[b]	3.95 ± 0.17	6.07 ± 0.35	3.61 ± 0.13	5.07 ± 0.20
Food intake *g/day*	25.20 ± 2.08[a]	49.20 ± 3.00	25.00 ± 1.26	47.40 ± 1.50	21.20 ± 0.86	46.40 ± 4.40
Water intake *mL/day*	47.00 ± 3.00[a]	330.00 ± 7.90	44.00 ± 4.80	252.00 ± 7.10	47.00 ± 7.68	334.00 ± 40.90
Urine volume *mL/day*	26.80 ± 3.26[a,c]	177.20 ± 8.90	32.80 ± 5.01	168.40 ± 5.00	31.80 ± 5.78	172.60 ± 3.60
Plasma glucose *mg/dL*	188 ± 10[a,c]	630 ± 7[d]	246 ± 14	771 ± 43	185 ± 7	638 ± 28
Urinary albumin *mg/day*	4.24 ± 0.31[a]	18.82 ± 0.87[d]	7.38 ± 0.18	33.30 ± 1.14	4.52 ± 0.30	15.86 ± 2.50

Each value represents the mean ± SEM. $N = 5$ rats in each group.
[a] Normal vs. diabetic (regular diet), $P < 0.001$
[b] Diabetic (regular diet) vs. diabetic (Se+ diet), $P < 0.05$
[c] Normal (regular diet) vs. normal (Se− diet), $P < 0.05$–0.001
[d] Diabetic (regular diet) vs. diabetic (Se− diet), $P < 0.05$–0.001

plasma glucose and urinary albumin levels in both normal and diabetic rats. In diabetic rats, Se supplementation did not normalize either plasma glucose or albuminuria. However, the kidney weight was significantly reduced by Se supplementation in diabetic rats. No effect of Se supplementation was observed on any of these parameters in normal rats.

Figure 1 demonstrates glomerular mRNA levels expressed as relative density units after normalization with β-actin for antioxidant enzymes in various groups of rats. The mRNA expression for Cu-Zn SOD and GSH-Px were significantly higher and that for catalase significantly lower in diabetic compared with normal rats. In Se-deficient rats, the mRNA expression for all these enzymes was significantly lower in both normal and diabetic rats compared with rats fed a regular diet. Supplementation of Se to diabetic rats significantly increased mRNA levels for all antioxidant enzymes compared with rats fed a regular or Se-deficient diets. Figure 2 shows representative autoradiograms of mRNAs for antioxidant enzymes in various groups of animals.

Figure 3 shows glomerular mRNA levels for TGF-β1. As evident, glomeruli from diabetic rats expressed more message than glomeruli from normal rats. Se deficiency caused a significant increase in glomerular expression of mRNA for TGF-β1 in normal rats, and this expression was even more pronounced in diabetic rats compared with rats fed a regular diet. Se supplementation significantly improved but did not normalize TGF-β1 expression in diabetic rats. However, Se supplementation had no effect in normal rats.

Glomerular volume and fractional mesangial area in normal and diabetic rats fed different diets are shown in Figure 4. As evident, no difference in glomerular volume was observed between normal and diabetic rats or rats fed either regular, Se-deficient, or Se-supplemented diets. However, the fractional mesangial area was significantly greater in diabetic compared with normal rats. Se deficiency significantly increased the mesangial area in both normal and diabetic rats. Se supplementation normalized this mesangial area in diabetic rats.

Figure 5 shows representative glomeruli from various groups of rats. Glomerular sclerosis is more prominent in diabetic than in normal rats fed a regular diet. Se deficiency increased sclerosis in both groups of rats. Supplementation of Se to diabetic rats reversed glomerular sclerosis in diabetic rats, but without any effect in normal rats.

Morphometric characteristics of interlobular arteries are shown in Table 4. As evident, diabetic rats had significantly increased total vessel area and decreased media:lumen ratio than normal rats. The decreased media:lumen ratio indicates an increase in lumen size in diabetic rats (Fig. 6). However, no difference either in media area, external diameter, or media thickness was observed between normal and diabetic rats fed a regular diet. In normal rats, Se-deficient diet caused a significant increase in the media:lumen ratio, suggesting a decrease in lumen size. In diabetic rats, Se deficiency significantly decreased total vessel area, media area, and external diameter, but increased media:lumen ratio without any effect on luminal area or media thickness. Although Se supplementation did not have any significant effect on the interlobular artery in normal rats, it had a profound beneficial effect in diabetic rats (Fig. 6). The total vessel and luminal areas and external diameter were significantly increased, whereas the media:lumen ratio was significantly reduced compared with diabetic rats fed a regular diet. However, both media area and thickness were not influenced by Se supplementation in diabetic rats.

Histologic examination of the tubulointerstitium showed

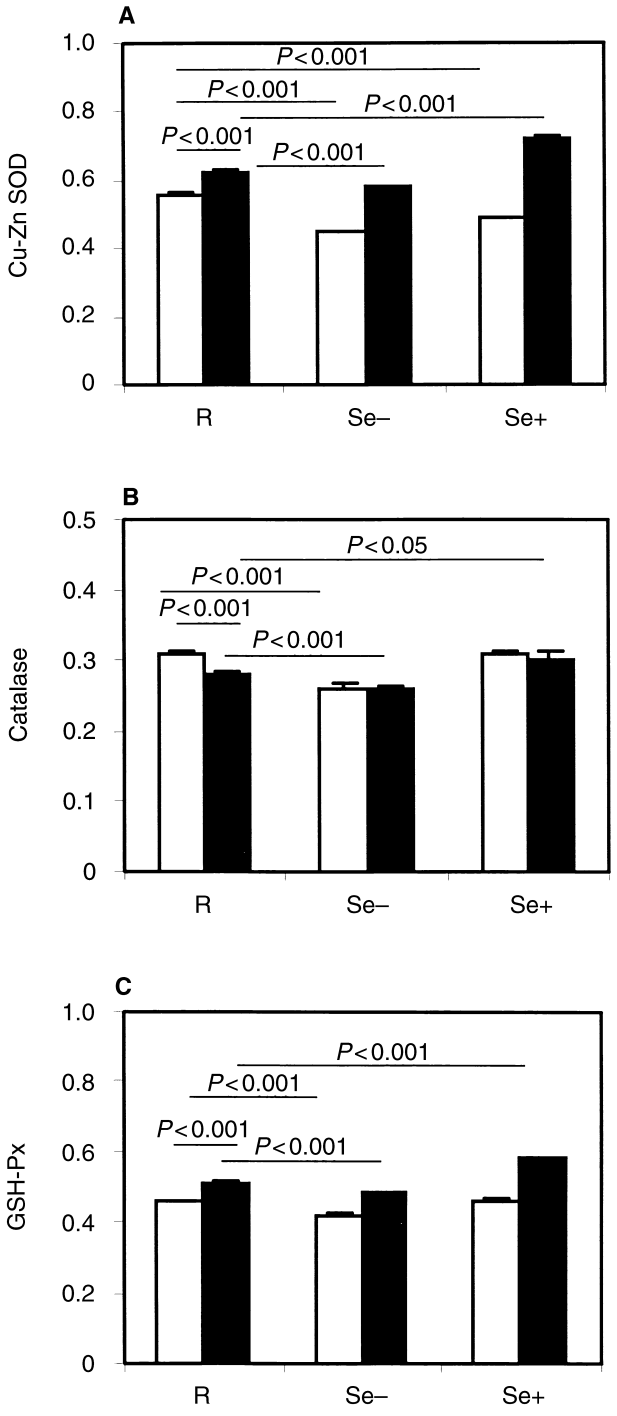


Fig. 1. RNase protection assays for glomerular mRNAs for Cu-Zn superoxide dismutase (Cu-Zn SOD), catalase, and glutathione peroxidase (GSH-Px) in normal (□) and diabetic (■) rats fed a regular (R) diet containing 0.27 mg/kg selenium (Se) or a Se-deficient (Se−) or a Se-supplemented (Se+; 0.78 mg/kg) diet for 10 weeks. Values were normalized to corresponding β-actin and expressed as relative density units. $*P < 0.001$ normal R vs. diabetic R. There were five animals in each group.

no abnormalities in normal rats fed either a regular, Se-deficient, or Se-supplemented diets. In contrast, diabetic rats fed a regular diet showed focal areas of tubular dilation, atrophy, and interstitial fibrosis (Fig. 7). Also, occasional rupture of the tubular basement membrane was observed in these diabetic rats (data not shown). The Se-deficient diet did not exacerbate any of these lesions; however, diabetic rats fed a Se-supplemented diet did not show any of the previously mentioned abnormalities in the tubulointerstitium. There were no inflammatory cellular infiltrates in any group of rats.

DISCUSSION

This study demonstrates several important observations. (*1*) TGF-β1 induces oxidative stress in vitro in the kidneys of both normal and diabetic rats, and this oxidative stress is abrogated by the antibody to TGF. (*2*) Similar to TGF-β1, Se deficiency induces oxidative stress in vivo in both normal and diabetic rats by reducing glomerular expression of mRNAs for antioxidant enzymes with an up-regulation of glomerular mRNA expression for TGF-β1. (*3*) Se deficiency increases albuminuria in both normal and diabetic rats, but renal growth in the diabetic rat only. (*4*) Se deficiency increases glomerular sclerosis and fractional mesangial area in normal and diabetic rats. (*5*) Se deficiency increases plasma glucose concentration in normal rats with further exacerbation in diabetic rats. (*6*) Se deficiency causes decreased lumen size of the interlobular artery in both normal and diabetic rats, and (*7*) Se supplementation to diabetic rats reduces the kidney weight, up-regulates antioxidant enzyme mRNAs, and significantly improves but does not normalize TGF-β1 mRNA levels. Also, Se supplementation not only reverses glomerular sclerosis, but also greatly enlarges the lumen size of the interlobular artery in these diabetic rats. However, plasma glucose levels and albuminuria were not significantly affected by Se supplementation in diabetic rats. Se supplementation did not have any effect in normal rats.

The induction of oxidative stress by TGF-β1 was reported in several in vitro studies [69–74]. First, TGF-β1 has been shown to produce an oxidant effect on pulmonary vascular endothelial cells that is capable of causing injury to the endothelium [69]. Second, TGF-β1 caused suppression of mRNA expression for SOD and catalase by rat hepatocytes [70] and that of catalase and GSH-Px by pancreatic β-cells [71]. Third, TGF-β1 has been shown to decrease catalase and GSH-Px activities in pancreatic β-cells [71] and vascular smooth muscle cells [72], and finally, TGF-β1 has been shown to induce hydrogen peroxide production by human lung fibroblasts [73] and hepatic stellate cells [74]. However, these studies did not utilize the neutralizing antibody to TGF-β to document the specificity of TGF-β1 effect. Our in vitro studies of

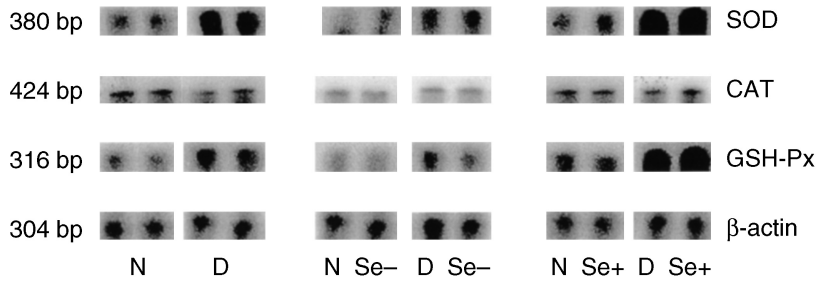


Fig. 2. Representative autoradiograms of mRNAs for Cu-Zn superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in normal (N), diabetic (D), selenium-deficient (Se−), or selenium-supplemented (Se+) rats.

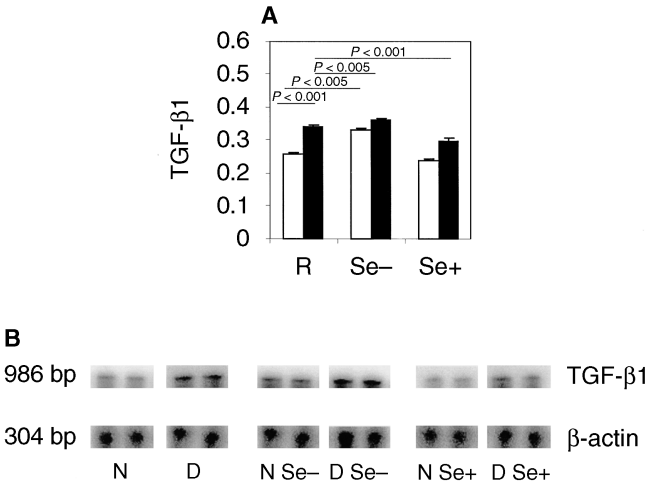
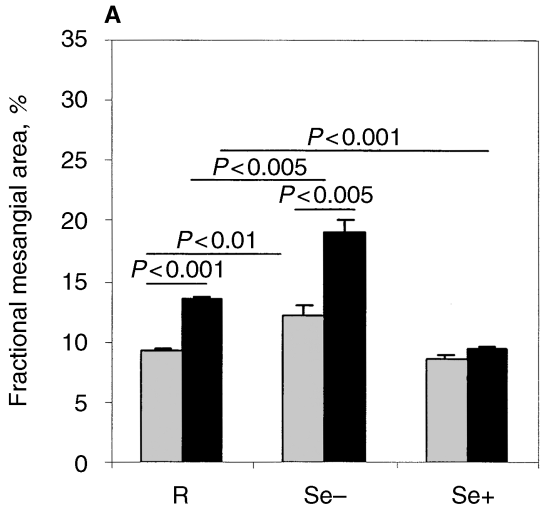


Fig. 3. RNase protection assay for glomerular mRNA for TGF-β1 expressed as density units (*A*) and representative autoradiograms (*B*) in normal (□; N) and diabetic (■; D) rats fed a diet containing either a regular (R) amount of selenium (Se) or a Se-deficient (Se−) or a Se-supplemented (Se+) diet for 10 weeks. Values were normalized to corresponding β-actin and are expressed as relative density units. $*P <$ 0.001 normal R vs. diabetic R. There were five animals in each group.



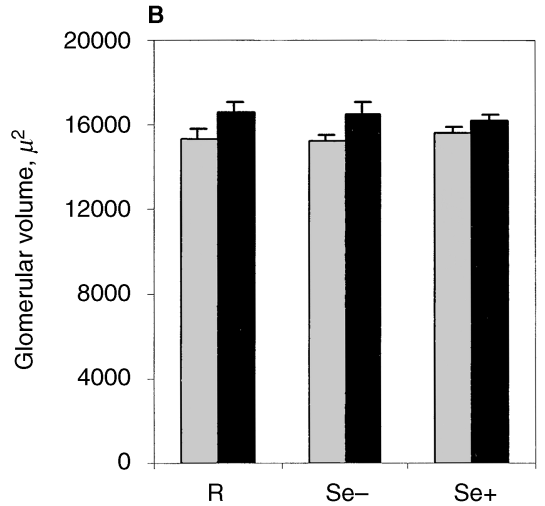


Fig. 4. Glomerular morphometry in normal (■) and diabetic (■) rats fed a diet containing either a regular (R) amount of selenium (Se) or a Se-deficient (Se−) or a Se-supplemented (Se+) diet for 10 weeks. $*P <$ 0.001 normal R vs. diabetic R. There were five animals in each group.

TGF-β1 and its antibody support these earlier studies and implicate this growth factor in the induction of oxidative stress.

To our knowledge, the observed increase in lipid peroxidation and the decrease in total glutathione content by TGF-β1 in both normal and diabetic glomeruli have not been reported previously. This increase in lipid peroxidation may be due to a decrease in GSH-Px activity induced by TGF-β1.

The oxidative stress induced by TGF-β1 has also been observed in Se-deficient normal and diabetic rats. Although diabetes caused up-regulation of Cu-Zn SOD and GSH-Px mRNA expression, Se deficiency caused down-regulation of these messages. These disparate effects are difficult to explain at this time; however, Se and hyperglycemia seem to play different roles in the transcriptional regulation of these enzymes. Thus, it is of interest to note that Se deficiency down-regulates rather than up-regulates the mRNA expression for antioxidant enzymes. Although Se deficiency increases the expres-

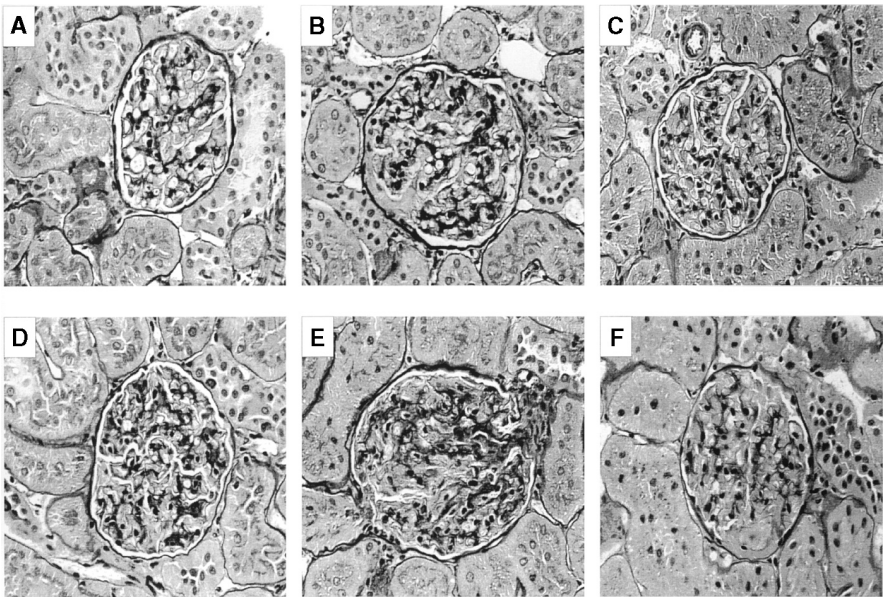


Fig. 5. Photomicrographs of representative glomeruli from rats fed either a regular, selenium-deficient (Se−), or Se-supplemented (Se+) diet for 10 weeks. (*A*) Normal rat fed a regular diet. (*B*) Normal rat fed a Se− diet. (*C*) Normal rat fed a Se+ diet. (*D*) Diabetic rat fed a regular diet. (*E*) Diabetic rat fed a Se− diet. (*F*) Diabetic rat fed a Se+ diet. Periodic acid-Schiff ×400.

Table 4. Characteristics of interlobular artery determined by morphometric study in normal and diabetic rats fed either a regular, selenium-deficient (Se−) or selenium-supplemented (Se+) diet

	Normal (5)	Diabetic (5)	Normal (5)	Diabetic (5)	Normal (5)	Diabetic (5)
Parameter	Regular		Se−		Se+	
Total vessel area μm^2	3857 ± 293^{a}	4616 ± 104^{e}	3800 ± 260	2972 ± 244	4244 ± 545^{c}	6981 ± 615^{f}
Luminal area μm	1072 ± 103	1280 ± 157	994 ± 102	1058 ± 259	1301 ± 293^{c}	2926 ± 323^{f}
Media area μm^2	1620 ± 243	2141 ± 188^{e}	1528 ± 249	1214 ± 126	1731 ± 243^{c}	2436 ± 150
External diameter μm	47.57 ± 2.02	55.25 ± 3.34^{e}	50.38 ± 2.37^{b}	44.13 ± 1.17	56.29 ± 3.46^{c}	72.29 ± 2.48^{f}
Media thickness μm	12.58 ± 0.84	11.90 ± 0.84	13.42 ± 1.50	11.40 ± 0.81	12.30 ± 0.97	12.80 ± 0.64
Media:lumen ratio	$0.51 \pm 0.03^{a,d}$	0.40 ± 0.03^{e}	0.68 ± 0.05	0.56 ± 0.03	0.43 ± 0.04^{c}	0.28 ± 0.02^{f}

Values shown are mean ± SEM. Numbers in parentheses represent number of animals in each group.
[a] Normal vs. diabetic (regular diet), $P < 0.05$–0.03
[b] Normal (regular diet) vs. diabetic (Se− diet), $P < 0.05$
[c] Normal vs. diabetic (Se+ diet), $P < 0.05$–0.01
[d] Normal (regular diet) vs. normal (Se− diet), $P < 0.03$
[e] Diabetic (regular diet) vs. diabetic (Se− diet), $P < 0.02$–0.001
[f] Diabetic (regular diet) vs. diabetic (Se+ diet), $P < 0.02$–0.01

sion of TGF-β1, the possible link between oxidative stress, and the molecular mechanism by which this growth factor reduces antioxidant gene expression remains to be elucidated. Our data of Se deficiency-induced up-regulation of TGF-β1 expression and down-regulation of antioxidant enzyme expression are consistent with those of Nath et al [38], who also demonstrated an increase in cortical expression of mRNA for TGF-β1 and a decrease in gene expression for GSH-Px and catalase in rats fed a diet deficient in Se and vitamin E. Also, decreases in hepatic and renal mRNA expression for GSH-Px have been observed in Se-deficient rats [21–23].

The mechanism by which Se deficiency causes proteinuria and glomerular sclerosis is not clearly understood. The results of the present study implicate TGF-β1 as the mediator of renal injury, because of the involvement of this growth factor in fibrogenesis [46–53] and proteinuria [75, 76]. Although Se supplementation did not normalize TGF-β1 mRNA expression and albuminuria in diabetic rats, glomerular sclerosis was normalized. This suggests that modest Se intake seems to counteract the fibrogenic action of TGF-β1, and normalization of this growth factor may take longer than 10 weeks of Se supplementation. Improvement in proteinuria by Se supplementation has been observed in animals [27, 34, 35] and human subjects [36]. In the present study, the observed dissociation between albuminuria and glomerular sclerosis is difficult to explain; however, small number of animals and variability in albumin levels may be responsible for this discrepancy. Although prevention of glomerular sclerosis in diabetic rats by Se supplementation has been reported by Douillet et al [35], the present study documents, to our knowledge for the first time, such a beneficial effect by morphometric evaluation. Also, Se supplementa-

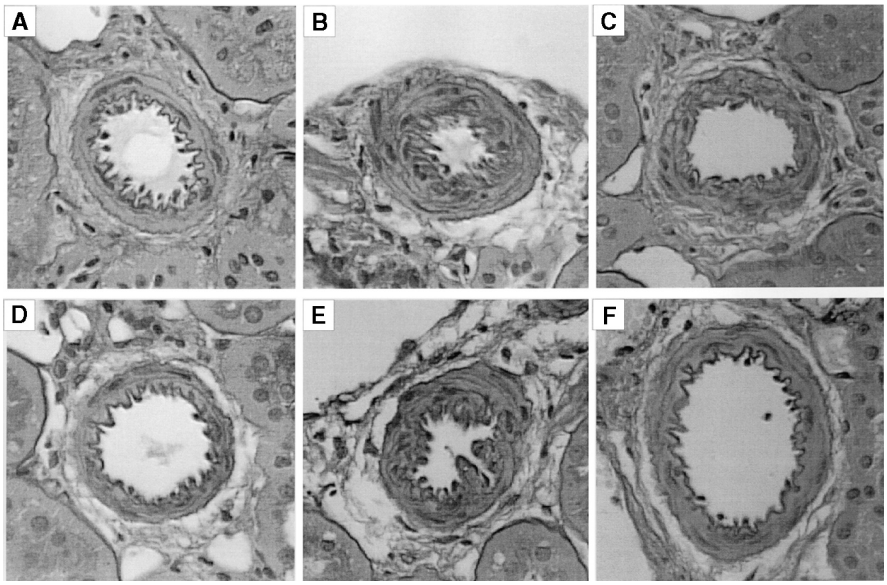


Fig. 6. Photomicrographs of representative interlobular arteries from rats fed either a regular, selenium-deficient (Se−), or selenium-supplemented (Se+) diet for 10 weeks. (*A*) Normal rat fed a regular diet. (*B*) Normal rat fed a Se− diet. (*C*) Normal rat fed a Se+ diet. (*D*) Diabetic rat fed a regular diet. (*E*) Diabetic rat fed a Se− diet. (*F*) Diabetic rat fed a Se+ diet. Periodic acid-Schiff ×400.

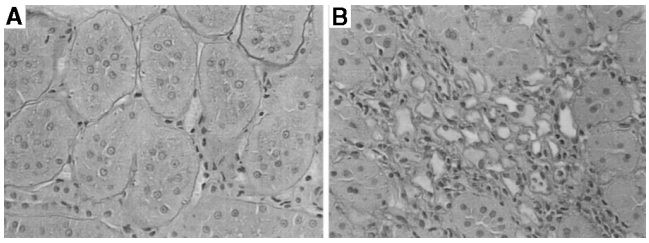


Fig. 7. Photomicrograph of the tubulointerstitium in normal (*A*) and diabetic (*B*) rats fed a regular diet. Periodic acid-Schiff ×400.

tion has been found to reduce the kidney weight in diabetic rats.

Selenium deficiency, like insulin deficiency, caused a significant increase in plasma glucose levels in normal rats with exacerbation in diabetic rats. This suggests that Se has insulin-like effects, and the observation is similar to that of other investigators [24–28]. However, Se supplementation failed to lower plasma glucose levels in diabetic rats. Probably lowering plasma glucose may take a longer duration of Se supplementation.

To our knowledge, this is the first report to evaluate morphometric characteristics of the renal artery in Se deficiency. Although the lumen size is larger in diabetic than in normal rats, Se deficiency caused a significant reduction in lumen size in both groups of rats. This suggests that Se deficiency may induce atherogenesis in normal as well as diabetic rats. It is interesting that Se supplementation caused a further increase not only in the total vessel area, but also in the lumen size in diabetic rats. This indicates that Se induces remodeling of the artery in these rats. The mechanism by which Se improves renal artery structure in diabetic rats remains unknown and warrants further investigation.

Although focal areas of tubulointerstitial disease were observed in diabetic rats, exacerbation of these lesions was not observed in rats fed a Se-deficient diet. This lack of effect of Se deficiency may be due to the short duration of feeding the deficient diet, since Nath et al [38] also failed to observe severe interstitial disease in rats fed a diet deficient in Se and vitamin E for 12 weeks. The observation that diabetic rats given excess Se in diet did not demonstrate any tubulointerstitial disease provides an indirect evidence that Se supplementation may be beneficial in preserving tubulointerstitium.

In summary, our data show that TGF-β1 is a pro-oxidant and that Se deficiency increases oxidative stress via this growth factor. In addition, Se deficiency may simulate hyperglycemic conditions. This conclusion is based on observations that both conditions increase TGF-β1 expression, induce oxidative stress, cause albuminuria and glomerular sclerosis, and elevate plasma glucose levels. Se supplementation to diabetic rats prevents not only oxidative stress but renal structural injury as well. Long-term studies with Se supplementation in diabetic rats are clearly indicated.

ACKNOWLEDGMENTS

This study was supported in part by the Kidney Research Foundation of New Jersey, Ruth Papier chapter and Dean's Biomedical Research Support Grant, UMDNJ-New Jersey Medical School, Newark, NJ, USA. We thank Dr. Venkata R. Nimmagadda for his help with morphometric studies.

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